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Enzyme-Linked Immunosorbent Assay of Ampicillin in Milk

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Abstract—An indirect immunoassay for quantitative determination of ampicillin (range, 10–1000 ng/ml) in buffer or milk has been developed. Polyclonal antibodies were obtained against ampicillin conjugated with bovine serum albumin; the conjugate was synthesized by direct condensation using carbodiimide. The antibodies were specific for ampicillin and exhibited low cross-reactivity to other penicillins (azlocillin, 17%; penicillin G, 10%; piperacillin, 5%; and carbenicillin, 4%). Matrix effects were minimized by combining the use of a casein-supplemented buffer (content of casein, 1%) with sample dilution. Limit of detection for ampicillin in milk (diluted tenfold) was equal to 5.0 ng/ml (which corresponded to 50 ng/ml of the original sample).

β-Lactam antibiotics, particularly penicillins (Fig. 1), are widely used in medicine and veterinary medicine, this being the reason why residual amounts of penicillins may be found in foodstuffs of animal origin. Antibiotics contained in milk may adversely affect the health of human consumers (e.g., by inducing allergic reactions and dysbacterioses). Moreover, the presence in milk of antibiotics and other compounds suppressing the development of microorganisms disrupts technological processes of production of cheese (including soft cheese) and sour milk beverages by retarding or blocking lactic acid fermentation. Therefore, milk should be carefully controlled for the presence of residual amounts of penicillins, and such control requires reliable and readily available analytical methods.

In Russia, maximum permissible levels (MPLs) for penicillins in milk have been established for penicillin G (the MPL is set to 0.01 U/g [1], which roughly corresponds to 5.9 μg/kg [2]). In countries of the European Community, MPLs are fixed for the following penicillin antibiotics: amoxycillin; ampicillin; penicillin G (4 μg/kg); and isoxazolyl penicillins (cloxacillin, oxacillin, and dicloxacillin) and nafcillin (30 μg/kg in each case) [3].

There are several methods whereby residual amounts of penicillins are determined in milk. Conventional microbiological methods, based on inhibition of growth of cultured microorganisms in the presence of antibiotics, usually exhibit sufficient sensitivity, but they are time-consuming [4]. A number of relatively rapid and simple microbiological tests have been launched recently that allow results to be obtained in two to three hours. One of them, known as Delvotest (DSM Food Sciences, the Netherlands), is highly sensitive to penicillins (e.g., the minimum detectable amounts of ampicillin and penicillin G are equal to 3 and 2.5 ng/ml). Delvotest allows detection of 30 antibacterials. However, the broad specificity of microbiological methods precludes identification of individual antibiotics, this being the reason why they are used for qualitative control of milk (screening for the presence of residual amounts of inhibitors, including antibiotics, sulfonamides, and disinfectants). Enzyme-based techniques are more rapid (the duration of one analysis approximates 20 min); this approach involves specific inhibition of certain enzymes in the presence of β-lactam antibiotics [5]. Due to a high selectivity, chromatographic methods are more suitable for identification; they are labor-intensive and more expensive than the methods described above [6].

Immunochemical methods of analysis, enzyme-linked immunosorbent assays (ELISAs) in particular, are used worldwide for determining residual amounts of antibiotics in foodstuffs of animal origin. As a rule, ELISAs are used as routine screening tests [7, 8]. These methods are more specific and rapid than microbiological or enzyme-based techniques. The use of ELISAs makes it possible to complete large-scale screening studies of samples within short periods of time. ELISAs are relatively cheap, and they do not require complicated sample preparation or sophisticated instrumentation.

ELISAs are widely used abroad for determining penicillin in milk [9–11], including as commercially available kits [12]. Determination of penicillin G, ampicillin, and isoxazolyl penicillins (cloxacillin and dicloxacillin) in milk has been described, with detection limits in the range 10–30 ng/ml [9–11]. A modification of an ELISA in which a fluorescent probe replaces conventional chromogens and capillaries are used for simultaneous determination of six penicillins in milk has been described [13]. Determination of residual penicillins in milk following cleavage of the β-lactam ring (which usually increases the sensitivity) has been the subject of several reports [11].

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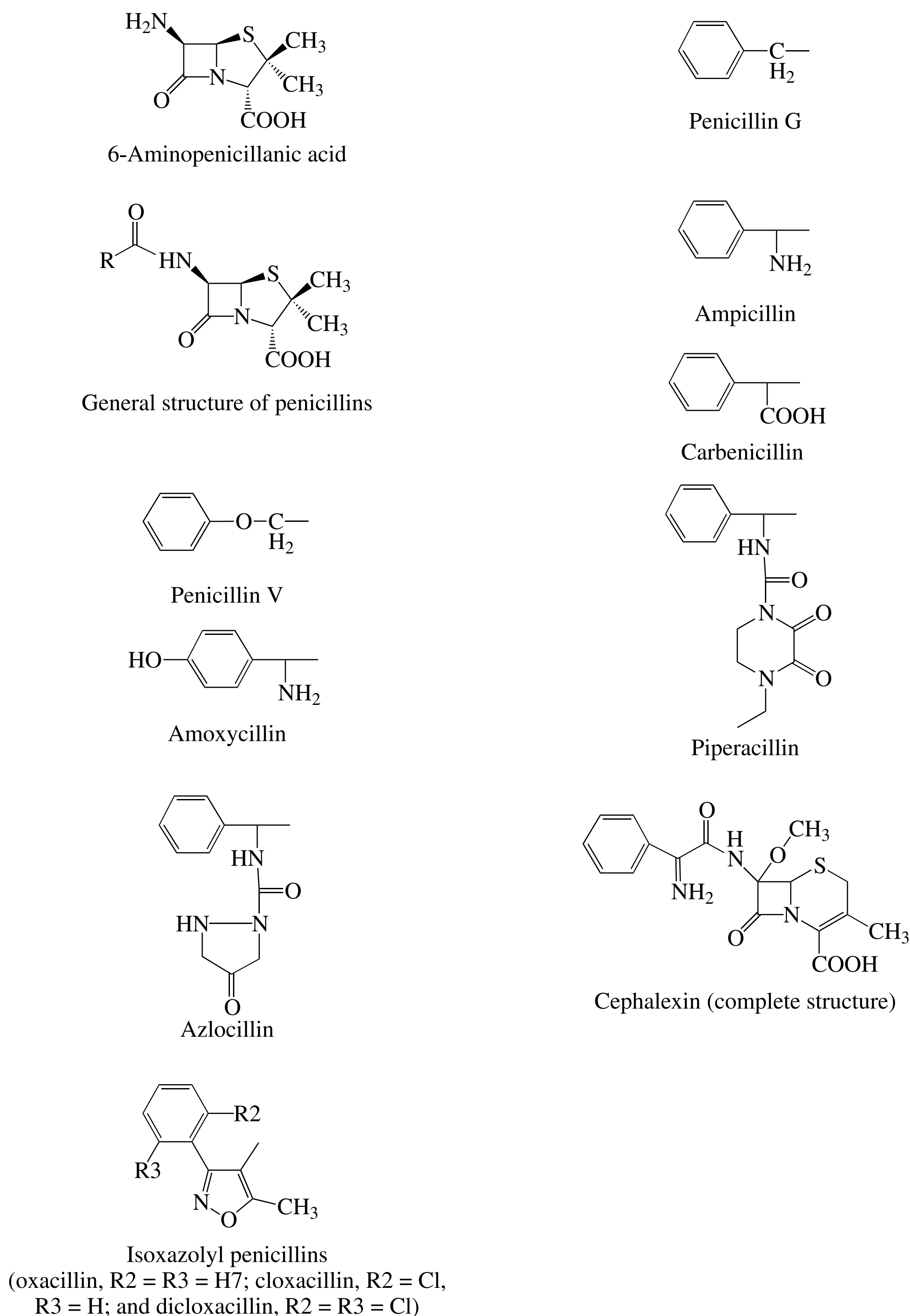

Fig. 1. Chemical structures of 6-aminopenicillanic acid and some penicillin antibiotics.

Attempts to shorten the time needed for performing analyses have prompted researchers to develop biosensor-based methods, which are viewed as an alternative to conventional ELISAs [14]. As a rule, biosensors are analytical devices combining biological materials (enzymes, cells, receptors, antibodies, etc.) with a transduction system for signals (electrochemical, piezoelectric, magnetic, or optical). Determination of ampicillin in milk using monoclonal antibodies and optical biosensors (a Biacore system, Sweden) has been

described [15]. To enable determination of ampicillin molecules with open β-lactam rings, milk samples were subjected to chemical or enzymatic treatment (the respective detection limits equaled 33 and 12.5 μg/l). The Biacore system served as a base for developing a penicillin G milk test (detection limit, 2.6 μg/l); a receptor protein exhibiting carboxypeptidase B activity served as the sensor molecule [16]. An amperometric sensor involving another receptor protein has been described, which is also appropriate for penicillin G determination in milk.

The prime task to be dealt with in developing an ELISA is to isolate and characterize immunospecific reagents. Antibodies are major reagents for any immunoassay. Multiple occurrences of allergic reactions to clinically administered β-lactam antibiotics provided an impetus for detailed studies of the immunological behavior of these low-molecular-weight compounds [18]. The use of hybridoma technology allowed identification and quantitation of antigenic determinants within the molecule of penicillin, in response to which antibodies are preferentially raised. The studies conducted made it possible to obtain monoclonal antibodies against at least three major epitopes: the thiazolidine ring, the side chain, and the new determinant generated on interaction of the carbonyl group of the β-lactam with an amino group of a protein [18–20].

The literature is replete with methods for obtaining poly- or monoclonal antibodies against penicillins with closed [10, 19, 21–23] or open [9, 11, 18–20, 23] β-lactam rings (group I and II immunogens, respectively). Preparation of group I immunogens involves their conjugation with proteins. Several methods have been published in which ampicillin is conjugated via its amino group [19, 21–23]; in addition, both ampicillin [19] and cloxacillin [10] may be conjugated via the carboxyl of the thiazolidine ring using the method of mixed anhydrides. Group II immunogens are obtained as described below. The β-lactam ring of penicillin is cleaved under alkaline conditions (pH 10 or 11), and the carbonyl formed is allowed to react with the protein amino group, resulting in the formation of an amide bond. Ring cleavage is associated with the loss of the antimicrobial activity, whereas the immunogenicity is preserved. Proteins conjugated with group II penicillins produce stronger immune responses than conjugates of their group I counterparts retaining the ring structure [21–23]. From a practical standpoint, however, antibodies against group II immunogens are of negligible value because MPLs are established for whole compounds [1, 3]. Moreover, the use of such antibodies in immunoassays requires that the target antibiotic in the sample be prehydrolyzed (chemically or enzymatically) in order to open the β-lactam ring, and this makes the analytical procedure longer.

It is not infrequent that the specificity of anti-penicillin antibodies is unpredictable. The antibodies are either highly specific [9] or cross-reactive with several closely related compounds [10, 19]. There are several reports describing methods of obtaining polyclonal [21] and monoclonal [22] antibodies with broader specificities, e.g., recognizing a common structural element—such as the β-lactam ring conjugated with the thiazolidine cycle—within a group of penicillin antibiotics.

Direct conjugation of ampicillin to proteins is a frequent approach to preparing immunogens for raising antibodies [19, 21]. As a rule, obtaining such conjugates does not constitute a complicated task because the target antibiotic contains a reactive amino group within its structure. Moreover, ampicillin is acid-resistant [2]. Its conjugation to proteins may involve the use of carbodiimide [23], glutaraldehyde [21–23], or the intermediate crosslinking agent 3-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) [19, 23]. Data on the immunogenicity of protein conjugates of ampicillin obtained by different approaches are rather controversial. It has been noted that the use of glutaraldehyde as a crosslinking agent produces weakly immunogenic conjugates [21, 22]. For example, administration of such a bovine serum albumin (BSA)–ampicillin conjugate to rabbits allowed isolation of specific antibodies from one animal after 13 weeks of immunization, whereas no specific antibodies formed in response to hemocyanin and glucose oxidase conjugates (also obtained using glutaraldehyde) [21]. In the case of monoclonal antibodies, conversely, a specific immune response was induced only by the hemocyanin conjugate [22]. Cliquet *et al.* [23] compared several methods of ampicillin conjugation to proteins. Immunization of mice for the purpose of producing monoclonal antibodies demonstrated that albumin–ampicillin conjugates obtained using carbodiimide failed to induce a specific response. With thyroglobulin conjugates (obtained in the same way), as well as BSA or ovalbumin conjugates (obtained using glutaraldehyde and MBS), specific antibody production was detected.

In this work, we sought to obtain and characterize immunoreagents (protein conjugates and polyclonal antisera) and develop a quantitative indirect ELISA for ampicillin determination in milk.

MATERIALS AND METHODS

Used in this work were ampicillin, penicillin G, tetracycline, streptomycin, chloramphenicol, BSA, casein, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 3',3',5,5'- tetramethylbenzidine, Tween 20, and complete Freund's adjuvant (Sigma, United States); dimethylsulfoxide (Lancaster, United Kingdom); secondary antibodies conjugated with horseradish peroxidase (Medgamal branch of the Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences); and high purity grade acids and inorganic salts (Khimmed, Russia). Azlocillin, piperacillin, carbenicillin, penicillin V, amoxycillin, oxacillin, cephalexin, 6-aminopenicillanic acid, gen-

tamicin, and erythromycin were provided by the State Research Center for Antibiotics (Moscow, Russia).

Milk (containing 0.5, 1.5, or 3.2% fat) was purchased in food stores of Moscow.

Standard solutions of ampicillin and other antibiotics were prepared by diluting stock solutions (1 mg/ml) with phosphate-buffered saline (PBS; 0.01 M potassium phosphate buffer, pH 7.4 supplemented with 0.15 M NaCl). Standard ampicillin solutions for milk analysis were prepared in PBS containing 1% casein.

Optical densities were measured using a Molecular Devices multichannel spectrophotometer for 96-well plates (United States).

Synthesis of protein conjugates of ampicillin. Ampicillin sodium salt and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (83 μmol each) were added to 2-ml aliquots of distilled water containing dissolved protein (0.83 μmol BSA or casein). The reaction mixtures were incubated at room temperature under continuous stirring for 2 h, followed by incubation at 4°C for 14 h. The levels of pH were maintained at 5.0 or 6.0 (for BSA and casein, respectively). The conjugates obtained were dialyzed against distilled water and lyophilized.

Obtaining polyclonal antisera. Rabbits were immunized with ampicillin–BSA according to the protocol below. A 1 : 1 mixture of the immunogen solution (1 mg/ml) and complete Freund's adjuvant was administered subcutaneously for one month at weekly intervals. Thereafter, during the three subsequent days, the solution of the immunogen (1 ml) was administered intravenously on a daily basis. Blood specimens were collected after one week. Repeated administration of the immunogen and blood specimen collection were performed every five or six weeks. The antisera obtained were tested by ELISA.

Enzyme-linked immunosorbent assay. A solution of the ampicillin–casein conjugate in 0.01 M sodium carbonate buffer (pH 9.6) was introduced into the wells of a 96-well polystyrene plate (Biohit, Finland), 250 μl/well. Following overnight incubation at 4°C, the plate was washed three times with PBS that contained 0.05% Tween 20 (PBST), 250 μl/well per wash cycle. Thereafter, 100 μl of standard ampicillin solution (0–1000 ng/ml) and 100 μl of antiserum solution in PBST were added into each well. Following incubation at 37°C for 1 h, the solution was removed, the wells were washed with PBST three times (250 μl/well per wash cycle), and 200 μl of a solution of the conjugate of secondary antibodies with horseradish peroxidase in PBST was added into each well. Following incubation at 37°C for 1 h, the solution was removed, the wells were washed with PBST three times (250 μl/well per wash cycle), and 200 μl of the buffer solution was added into each well. The buffer solution contained 25 ml of 0.1 M acetate buffer (pH 5.5), 400 μl of a 6 mg/ml solution of 3',3',5,5'-tetramethylbenzidine in dimethylsulfoxide, and 3 μl of 30% hydrogen peroxide. The reaction was stopped after 10–15 min by adding 50 μl/well 4 M H_2SO_4 solution, and the optical density was recorded at 450 nm.

Samples for analysis were prepared by dissolving ampicillin in milk with variable fat content. Thereafter, the samples were defatted by centrifugation (2000 *g*, 15 min) and the aqueous phase ("as is" or diluted tenfold by PBS supplemented with 1% casein) was analyzed by ELISA as described above, with the following modifications: (1) the volume of standard ampicillin solutions (or samples for analysis) was 50 μl and (2) the volume of the solution of the antiserum (in PBS supplemented with 1% casein) was 150 μl.

RESULTS AND DISCUSSION

In this work, we performed a simple single-stage conjugation of ampicillin to BSA (for immunization) or casein (for adsorption to the wells of ELISA plates) using soluble carbodiimide. Polyclonal antisera (obtained as a result of immunization of four experimental animals) were tested by ELISA for the ability to bind the ampicillin–casein conjugate adsorbed to the solid phase. The values of 50% titers increased from 1/2000–1/4000 (the first immunization cycles) to 1/30000–1/60000 (starting from the third immunization cycle in the most reactive animal). The values of the titers were indicative of the presence in the antisera of high concentrations of antibodies recognizing ampicillin within its protein conjugate. The results obtained clearly demonstrated that the ampicillin–BSA conjugate prepared using carbodiimide was capable of generating a specific immune response, contrary to prior reports [23].

The development of the new ELISA involved several stages. Particular attention was given to the choice of the antiserum, which was to combine two features: (1) the presence of specific antibodies at high concentrations and (2) a high affinity of the antibodies. In all four animals, both the titers of the antibodies and their affinity increased throughout the immunization, which ensured the high sensitivity of the assay. Figure 2 shows the calibration curves obtained after optimization of the conditions of the analysis for each antiserum corresponding to a cycle of immunization of the most reactive animal. The optimization made it possible to develop an ELISA for detecting ampicillin in buffer solutions. The detection limit equaled 5.1 ng/ml; ampicillin could be detected within the range 10–1000 ng/ml (Fig. 2, curve *5*). The method was highly reproducible. CV values for solutions containing 3.3, 33.3, and 333 ng/ml ampicillin amounted to 8.2, 9.8, and 6.0%, respectively, for intra-assay reproducibility ($N = 10$, $P = 0.95$), or, in the case of interassay reproducibility experiments ($N = 3$, $P = 0.95$), to 10.3, 5.9, and 4.6%, respectively.

The assay developed was ampicillin-specific (Table 1). Among the compounds of similar structure, only azlocillin, penicillin G, piperacillin, and carbenicillin

exhibited cross-reactivity in excess of 0.1%. Azlocillin was the most active in this respect (17%). Of note, cross-reactivities were exhibited by substances with maximum similarity to ampicillin (penicillin G differs by the absence of an amino group and carbenicillin has a carboxy group instead of the amino group; Fig. 1). The ability of the antibodies to recognize azlocillin and piperacillin may be accounted for by the presence within their structures of bulky substituents and amide bonds, which mimic a portion of the ampicillin–protein conjugate used for immunization (Fig. 1). The cross-reactivities of antibiotics belonging to other families (gentamicin, streptomycin, tetracycline, chloramphenicol, and erythromycin) were below 0.1%. The profiles of specificity of the antibodies we obtained differ slightly from those reported in the literature for antibodies produced in response to similar ampicillin–protein conjugates [21, 22]. As a rule, such antibodies have a broader specificity. For example, polyclonal antibodies against an ampicillin–BSA conjugate synthesized using glutaraldehyde exhibited cross-reactivities to 16 penicillins in the range 27–355% (the reactivity with ampicillin was taken to be equal to 100%) [21]. Monoclonal antibodies obtained using a similar hemocyanin conjugate exhibited cross-reactivities to 13 penicillins in the range 10–160% (the reactivity with ampicillin was taken to be equal to 100%) [22].

At the next stage, we optimized the assay for determining ampicillin in milk with variable fat content. In developing the assay, we used data from the literature for orientation because of the lack of established MPLs for ampicillin in milk. Particular attention was given to studies of matrix effects of milk specimens on the analytical system under development. Approaches to development of an ELISA applicable to determining antibiotics (chloramphenicol) in milk were defined in detail in a prior work [24]. In order to counter the matrix effect, we combined sample dilution with the use of a buffer containing casein (the major milk protein) for plotting the calibration curve and diluting the samples. Parameters optimized in this work included (1) the sample to antibody ratio and (2) the composition of the buffer for reagent preparation and sample dilution; the goal was to retain the sensitivity of the assay and to eliminate the effects of components of the sample (milk constituents) on the behavior of the system. For this, we used specimens of sterilized or pasteurized milk with variable fat content (0.5, 1.5, or 3.2%). Prior to the assay, the test samples were defatted by centrifugation. Because the use of 100-μl aliquots of milk caused the optical density to decrease considerably, we decreased the volume of the sample to 50 μl. In this variant of the setting, the sensitivity of the assay was retained (on decreasing the volume of the sample further, a decrease in the sensitivity was observed; data not shown).

The combination of (1) decreased sample volume, (2) milk defatting, and (3) the use of a buffer supplemented with 1% casein (for preparing standard solutions and the solution of the specific antibodies) did not eliminate the matrix effect completely (Fig. 3). A direct relationship of the appearance of the calibration curve to the content of fat was still clearly traceable. It is generally held that, in analyzing real samples, it is better not to bring matrix components into contact with the enzyme because its activity may be affected. An indirect ELISA setting makes it possible to separate the immunological reaction (antigen–antibody interaction) from the stage at which the enzyme is used. However, the results obtained demonstrated that the matrix of the sample (the content of fat in particular) likely affected

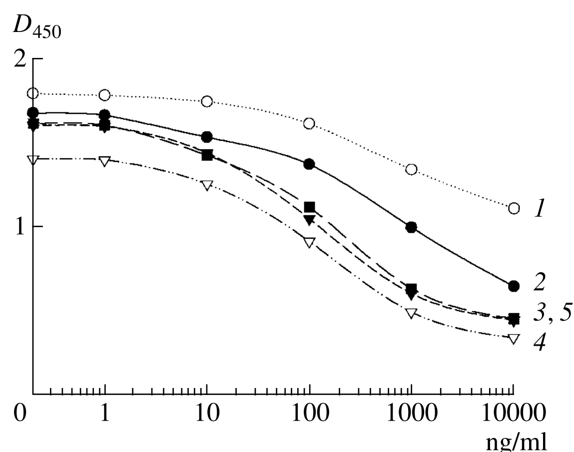

Fig. 2. Calibration curves for ELISA of ampicillin (ng/ml) in a buffer obtained using antisera of various immunization cycles isolated from the most reactive animal. Numbers of the curves (*1–5*) correspond to immunization cycles.

Table 1. Specificity of the polyclonal antibody–based ELISA

Compound	IC_{50}, ng/ml*	Cross-reactivity, %**
Ampicillin	62	100
Azlocillin	365	17
Penicillin G	620	10
Piperacillin	1240	5
Carbenicillin	1550	4
Penicillin V	>62000	<0.1
Amoxycillin	>62000	<0.1
Oxacillin;	>62000	<0.1
Cephalexin	>62000	<0.1
6-Aminopenicillanic acid	>62000	<0.1

* IC_{50}, concentration of a compound effecting a 50% inhibition of antibody binding to the solid-phase antigen.

** The percentages of cross-reactivity were calculated according to the formula: $\% = C_1/C_2 \times 100\%$, where C_1 and C_2 are IC_{50} values for ampicillin and the cross-reactive compound, respectively.

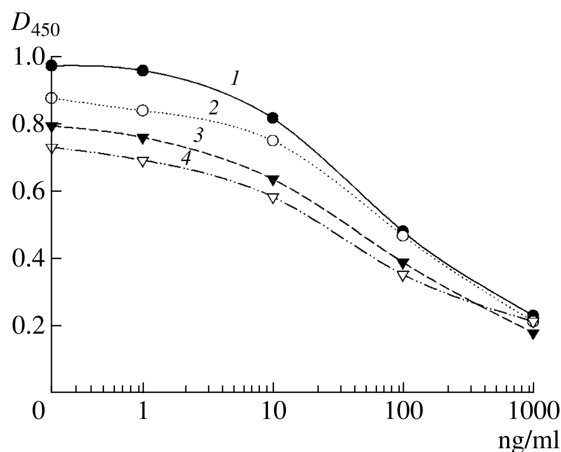

Fig. 3. Effect of fat content in milk on ELISA of ampicillin. Standard solutions of ampicillin were prepared using a buffer containing 1% casein (*1*), sterilized milk with 1.5% fat content (*2*), sterilized milk with 3.2% fat content (*3*), and pasteurized milk with 3.2% fat content (*4*).

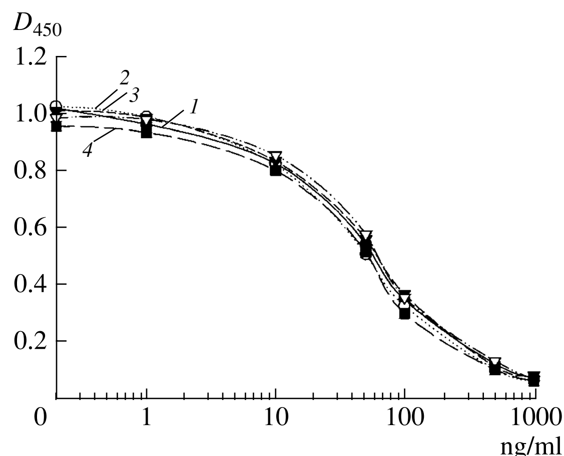

Fig. 4. Calibration curves for ELISA of ampicillin (ng/ml) in a buffer containing 1% casein (*1*) or milk with variable fat content: (*2*) 0.5, (*3*) 1.5, or (*4*) 3.2%.

the interaction of the specific antibodies with either the ampicillin–protein conjugate or the secondary antibodies–horseradish peroxidase conjugate, rather than the activity of the enzyme.

A series of experiments conducted to clarify the situation showed that the matrix effect was minimized by introducing an additional sample preparation step during which the sample was diluted tenfold with a buffer containing 1% casein (Fig. 4). This decreased the sensitivity but also eliminated the difference between milk specimens, making it possible to detect experimental contamination with the antibiotic (Table 2). In the majority of the samples assayed, ampicillin recovery ranged from 81 to 121%, although the values obtained with pasteurized milk were higher (114–171%). Thus, we succeeded in establishing conditions favoring ELISA determination of ampicillin in milk samples regardless of their composition. The method was highly reproducible (CV values did not exceed 15%). The detection limit equaled 5.0 ng/ml (milk diluted ten-

Table 2. Results of ELISA determination of ampicillin in milk with variable fat content

Sample	Ampicillin concentration, ng/ml		Recovery, %
	introduced	measured	
Sterilized milk (0.5% fat), milk factory, Moscow	0	<50	–
	100	107	107 ± 13
	500	591	118 ± 17
	1000	1210	121 ± 10
Sterilized milk (1.5% fat), milk factory, Moscow	0	<50	–
	100	95	95 ± 10
	500	473	95 ± 9
	1000	944	94 ± 17
Sterilized milk (3.2% fat), milk factory, Moscow	0	<50	–
	100	81	81 ± 10
	500	452	90 ± 10
	1000	978	98 ± 17
Pasteurized milk (3.2% fat), milk factory, Moscow	0	<50	–
	100	136	136 ± 15
	500	569	114 ± 14
	1000	1712	171 ± 12

fold), which corresponded to 50 ng/ml in the case of the original (undiluted) samples. This result is somewhat less optimistic than the data reported in the literature for penicillins [9–11]. It would seem that the sensitivity of the assay may be increased by refining the choice of reagents and modifying the settings of the procedure.

In assaying considerable amounts of samples, it becomes important to choose a method that would be appropriate for their storage prior to the test. The duration of the storage may vary from days to weeks. Freezing of the samples is the simplest way of storing them. In this case, however, thawing may change the structure of the sample, causing the protein components to undergo denaturing, with the resulting clotting and stratification of milk.

Effects of freezing methods on the outcome of the ELISA were assessed as described below. For each sample of milk containing a variable amount of fat (0.5, 1.5, or 3.2%), two series of standard solutions were prepared involving fresh or thawed milk, which were analyzed by ELISA against the calibration curve plotted using the buffer with 1% casein. Note that all calibration curves were identical (data not shown). Thus, sample freezing barely if at all affects ELISA results and may be recommended as a method of storage.

In conclusion, our study allowed us to (1) obtain polyclonal antibodies with a new spectrum of specificities and (2) select and optimize conditions of a specific ELISA for detecting residual amounts of ampicillin in milk specimens with variable fat content. The use of a buffer supplemented with 1% casein in combination with dilution of the samples minimized the matrix effect.

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